

LARVAL SURVIVAL AND GROWTH TEST WITH FATHEAD MINNOWS
(Pimephales promelas)

1. TEST OBJECTIVE

To assess the toxicity of a test material to *Pimephales promelas* and determine the effects on survival and growth (as determined by dry weight) of test animals compared to controls.

2. TEST ARTICLE

2.1 Description/Identification

Unless otherwise specified, the test material is supplied by the client. Adequate chemical specifications with special reference to hazardous properties and storage conditions are also supplied by the client.

2.2 Methods of Synthesis

In most cases, the test article is an effluent sample. Information on the methods of synthesis, stability, and composition--or other characteristics which define the test article--are on file with the client.

3. EXPERIMENTAL DESIGN

3.1 Test Organisms

3.1.1 Species

The test species is the fathead minnow, *Pimephales promelas*.

3.1.2 Source

P. promelas used for toxicity tests are usually obtained from stock cultures maintained at EA's Culture Facility. However, organisms may be obtained from a scientific organism vendor, if necessary.

3.1.3 Culturing and Holding Conditions

P. promelas brood stock are maintained at $25 \pm 2^\circ\text{C}$ and a 16-hour light, 8-hour dark photoperiod cycle in an environmentally controlled laboratory. Brood organisms are maintained in all glass aquaria in a recirculating system of dechlorinated municipal tap water. The eggs are held at $25 \pm 2^\circ\text{C}$ throughout hatching. If eggs are obtained from a scientific vendor, the eggs, upon receipt, are transferred to static recirculating holding tanks of an appropriate size containing dechlorinated tap water at the temperature ($\pm 2^\circ\text{C}$) of the water in which the organisms were shipped. The eggs are slowly elevated to $25 \pm 2^\circ\text{C}$ and examined regularly. Dead eggs, or those which appear unusually colored or malformed, are removed as observed, and recorded on appropriate log sheets. Certain regulatory or project specific objectives may require organism acclimation to the dilution water when it is different from the holding/culture water.

3.1.4 Age of Test Organisms at Test Initiation

Fathead minnow larvae (<24 hours old) are used for chronic toxicity testing. Under special circumstances (e.g., if eggs must be obtained from a scientific vendor), larvae used for chronic testing may be <48 hours old all hatched within a 24-hour window.

3.2 Dilution Water

The source of dechlorinated tap water is the City of Baltimore municipal water system. Upon entry to the laboratory, the water passes through a high-capacity, activated carbon filtration system to remove chlorine and other possible organic contaminants. This water source has proven safe for aquatic organism toxicity testing at EA, as evidenced by maintenance of multigeneration *Daphnia* sp. and fathead minnow cultures with no evident loss of fecundity. Reconstituted fresh water or other dilution water may be used depending on study requirements.

3.3 Test Concentration Series

The test concentration series consists of a minimum of five dilutions (e.g., 6.25, 12.5, 25, 50, and 100 percent effluent plus a control) and may be determined from a prior screening of the test material. Rangefinding assays utilize more widely spaced test concentrations and a control. Ambient water or effluent samples may also be evaluated as single concentrations and compared to a control.

3.4 Test Concentration Preparation

Test concentrations are prepared with Class A glassware.

3.5 Test Vessels and Test Volume

Test vessels are 1-L beakers; the final test volume is 250 ml. Other test vessels may be used depending on the study's requirements.

3.6 Test Organism Number

Tests are conducted using a minimum of three replicates, and as a standard practice four replicates, per concentration, with 10 organisms per container. Fish are randomly assigned to each replicate test container. More replicates can be added, if appropriate.

3.7 Test Environment

The test vessels are maintained in an environmentally controlled laboratory with a 16-hour light, 8-hour dark photoperiod. Temperature within the environmental room is monitored continuously using temperature recorders and is maintained at $25 \pm 1^\circ\text{C}$ (unless a different project--specific temperature is required).

3.8 Analysis of Test Concentrations for Test Article

If required, test solutions may be analyzed for verification of chemical concentrations. The analytical method and number of analyses are determined after consultation with the client. When chemical analyses are necessary, both nominal and actual measured test concentrations are reported.

3.9 Test Observations

Each test day, test organisms are observed to record the number of surviving organisms. Dead organisms are removed when observed. The study terminates after 7 days. The study may be extended, however, at the request of the client.

Each effluent or receiving water sample received is analyzed for temperature, conductivity, alkalinity, hardness, and total residual chlorine. Aliquots of effluent and receiving water may be gently aerated (100 bubbles/min) prior to test initiation if dissolved oxygen is less than 4 mg/L or greater than 100 percent saturation. After test initiation, if the dissolved oxygen in any test chamber is less than 4 mg/L, all test chambers are gently aerated or other corrective action is taken. Water quality measurements recorded daily on old and new test solutions include dissolved oxygen, pH, temperature, and conductivity from a minimum of one replicate of every concentration. Analytical determinations are conducted according to APHA et al. (1995) and

US EPA (1979).

At the end of the test period, surviving fish are rinsed and placed in pre-weighed, oven dried aluminum pans (one replicate per pan). Fish are oven dried for a minimum of 6 hours at 100°C after which each pan is weighed. The mean dry weight of the fish (weight of pan and fry minus weight of pan/number of fish) is calculated.

3.10 Solution Renewal (When Applicable)

The test solution is renewed daily. New solutions are prepared on the day of renewal. After the new solutions have reached test temperature and water quality measurements (temperature, pH, dissolved oxygen, and conductivity) are completed, the solution renewal may be performed by transferring the organisms from one test chamber to another or by replacing the test solution. If the test solution replacement method is used, caution must be given not to stress the test organisms while the test chamber is siphoned. Usually 75 percent of the old solution is removed and replaced; however, the amount is dependent on the size of the test organisms. The larvae in each test chamber are fed 0.1 ml (approximately 700 to 1,000) of a concentrated suspension of newly hatched (<24 hours old) brine shrimp nauplii (*Artemia sp.*) three times daily (morning, early afternoon, and the end of the work day). The nauplii are rinsed with dechlorinated tap water before feeding.

3.11 Data Analysis

Statistical analyses are performed on percent survival and mean dry weight data. Analysis of variance (ANOVA) and either Bonferroni's T-test or Dunnett's Mean Comparison test are used to analyze the survival and mean weight data for significance of effects. Depending on the distributional characteristics of the data generated, it may be necessary to use Steele's Many-One Rank Test or the Wilcoxon Rank Sum Test instead (US EPA 1994). The Shapiro-Wilks test (for datasets with ≤50 datapoints) or the Chi-Square test is used to test for normality of the reproduction data. Bartlett's test is used to test for homogeneity of variance of the reproduction data. If requested before the initiation of the study, growth data will be analyzed using EPA's ICp program to determine an IC50 and/or IC25. Although not standard practice, an LC50 may be calculated using the probit, moving average, and binomial methods as described by Stephan (1977). Depending on the nature of the data, other methods may be used, including the probit approximation method of Litchfield and Wilcoxon (1949), SAS probit analysis (SAS Institute 1985) or graphical interpolation using the log concentration vs. percent lethality as described by APHA et al. (1995). The methods used are specified in the final report.

3.12 Test Acceptability

An individual test may be conditionally acceptable if temperature, dissolved oxygen, and other specified conditions fall outside specifications, depending on the degree of the departure and the objectives of the tests.

4. FINAL REPORT

The final report is prepared to contain, at a minimum, the following information:

- . Objectives and procedures stated in the approved protocol, including any changes made to the original protocol
- . Identity of the test article(s) by name or code number and their strength (i.e., quality/purity), and a description of any pretreatment
- . Source of the dilution water, its chemical characteristics, and a description of any pretreatment
- . Test concentration series used and duration of the assay
- . Mean dry weights of test fish with the respective standard deviations
- . Water quality characteristics (pH, dissolved oxygen, temperature, etc.) of dilution water and selected test concentrations during testing
- . Any unforeseen circumstances that may have affected the quality or integrity of the study
- . Signature of the project manager, senior technical reviewer, and quality control officer authorizing release of the report
- . Location of all archived data and the original copy of the final report at EA

Items of data to be included in the report consist of experimental design and test performance, effects on general appearance of test organisms (if applicable), morbidity and mortality, presentation of water quality characteristics, survival and growth data.

5. QUALITY ASSURANCE

5.1 Amendments to Protocol

Amendments to the authorized protocol established by EA or by the client are made only after proper authorization. Such authorization is achieved by completion of the Protocol Amendment Form by EA after consultation with the client.

5.2 Standard Operating Procedures

Unless otherwise specified, all procedures given in the protocol are subject to detailed Standard Operating Procedures (SOPs) which are contained in the SOP manuals of the participating departments. These SOPs and protocols generally follow the types of requirements outlined in the U.S. EPA's Good Laboratory Practice Standards (GLPs) (US EPA 1989).

5.3 Reference Toxicant

A reference toxicant test, utilizing sodium dodecyl sulfate (SDS), cadmium chloride, or another appropriate chemical is used as an internal quality check of the sensitivity of the test organisms. Testing is conducted at least once monthly on organisms which are cultured in-house, and on each population of organisms purchased for testing from an outside source if reference toxicant data are not available from the supplier on the acquired lot. The results of each test are compared with historical, species-specific toxicological information from reference toxicant tests performed at EA to determine if the results are within acceptable limits. Limits are established using the control charts outlined in US EPA (1994).

5.4 Quality Assurance Evaluation

Studies conducted under this protocol may be subject to internal audit by EA's Quality Assurance Unit. A quality control officer is responsible for monitoring each study to assure the client that the facilities, equipment, personnel, methods, practices, records, and controls are in conformance with EA's QC program and, if applicable, EPA's GLPs.

5.5 Inspection by Regulatory Authorities

In the event of an inspection of EA by an outside authority during the course of the study, the client whose study is being inspected will be consulted before examiners are permitted access to any of the project records or the experimental areas.

5.6 Archives

Copies of project-specific records shall be transferred to the client promptly after the project is completed or as negotiated and budgeted. Original primary data are retained at EA for 5 years. Primary data include chain-of-custody records, laboratory data sheets, records, memoranda, notes, photographs, microfilm, and computer printouts that are a result of the original observations and activities of the study and which are necessary for the reconstruction and evaluation of the study report.

5.7 Location

Studies are conducted at the Ecotoxicology Laboratory of EA Engineering, Science, and Technology, Inc. at the Loveton Office in Sparks, Maryland.

6. SPECIFICATIONS OF THE FATHEAD MINNOW SURVIVAL AND GROWTH TOXICITY TEST

6.1 Basic References

American Public Health Association (APHA), American Waterworks Association, Water Environment Federation. 1995. Standard Methods for Examination of Water and Wastewater, 19th or most recent version. APHA, Washington, D.C.

EA. 1996. Quality Control and Standard Operating Procedures Manual for EA's Ecotoxicology Laboratory. Fifth Revision. EA Manual ATS-102. Internal document prepared by EA's Ecotoxicology Laboratory, EA Engineering, Science, and Technology, Inc., Sparks, Maryland.

Litchfield, J.T., and F. Wilcoxon. 1949. A simplified method of Evaluating Dose/Effect Experiments. J. Pharmacol. Exp. Ther. 96:99-113.

SAS Institute Inc. SAS Users Guide: Statistics, Version 5 Edition. Cary, NC:SAS Institute Inc., 1985.

Stephan, C.E. 1977. Methods of Calculating an LC50 in Aquatic Toxicology and Hazard Evaluation (F.L. Mayer and J.L. Hamlink, Eds.), pp. 65-84. ASTM STP 634, ASTM, Philadelphia, Pennsylvania.

US EPA. 1979. Methods for Chemical Analysis of Water and Wastes. EPA/600/4-79/020. U.S. Environmental Protection Agency, Washington, D.C.

US EPA. 1989. Toxic Substances Control Act (TSCA); Good Laboratory Practice Standards. Title 40 CFR Part 792. Fed. Regist. 54(158): 34034-34074.

US EPA. 1989. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms. Second Edition. EPA/600/4-89/001. U.S. Environmental Protection Agency, Environmental Monitoring Systems Laboratory, Cincinnati, Ohio.

US EPA. 1994. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms. Third Edition. EPA/600/4-91/002. U.S. Environmental Protection Agency, Environmental Monitoring Systems Laboratory, Cincinnati, Ohio.

6.2 Test Specifications

Test organism:	Fathead minnow (<i>Pimephales promelas</i>)
Temperature:	25±1°C
Organism age:	Preferably <24 hours old. If eggs are obtained from scientific vendor, larvae may be <48 hours old (24-hour window) at test initiation
Aeration:	None, unless dissolved oxygen falls below 4 mg/L
Light quality:	Wide-spectrum fluorescent light
Light intensity:	50-100 f.c.
Photoperiod:	16-hour light, 8-hour dark
Dilution water:	Dechlorinated municipal tap water, reconstituted fresh water, or appropriate receiving water
Test containers:	1-L beaker
Test volume:	250 ml per replicate
No. of concentrations:	Definitive assay - Minimum of five test concentrations and a control

	Screening assay - Single test concentration and a control
No. of replicates:	4 (minimum of 3)
No. organisms per replicate:	10
Feeding regime:	Feed 0.1 ml newly hatched (<24-hour old) brine shrimp nauplii three times daily (morning, afternoon, evening).
Test type and duration:	Static renewal, test duration 7 days
Endpoints:	Survival and growth
Test acceptability:	80 percent or greater survival in the control solution; average dry weight of surviving controls equals or exceeds 0.25 mg.